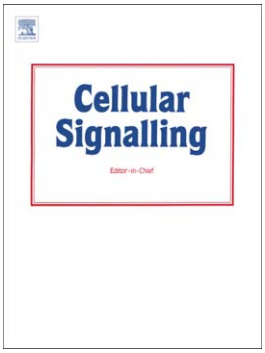
Accepted Manuscript



Power-Law Models of Signal Transduction Pathways

Julio Vera, Eva Balsa-Canto, Peter Wellstead, Julio R. Banga, Olaf Wolkenhauer

PII: S0898-6568(07)00046-0
DOI: doi: 10.1016/j.cellsig.2007.01.029
Reference: CLS 6380

To appear in: *Cellular Signalling*

Received date: 6 December 2006
Accepted date: 22 January 2007

Please cite this article as: Vera Julio, Balsa-Canto Eva, Wellstead Peter, Banga Julio R., Wolkenhauer Olaf, Power-Law Models of Signal Transduction Pathways, *Cellular Signalling* (2007), doi: 10.1016/j.cellsig.2007.01.029

Power-Law Models of Signal Transduction Pathways

Julio Vera[a], Eva Balsa-Canto[b], Peter Wellstead[c], Julio R. Banga[b], Olaf Wolkenhauer[a, 1]

[a]*Systems Biology and Bioinformatics Group. Department of Computer Sciences. University of Rostock. Rostock, Germany*

[b]*Process Engineering Group. Instituto de Investigaciones Marinas, Spanish Council for Scientific Research (CSIC). Vigo, Spain*

[c]*Hamilton Institute. National University of Ireland, Maynooth, Ireland*

16-Feb-07

Abstract

The mathematical modelling of signal transduction pathways has become a valuable aid to understanding the complex interactions involved in intracellular signalling mechanisms. An important aspect of the mathematical modelling process is the selection of the model type and structure. Until recently, the convention has been to use a standard kinetic model, often with the Michaelis-Menten steady state assumption. However this model form, although valuable, is only one of a number of choices, and the aim of this article is to consider the mathematical structure and essential features of an alternative model form – the power-law model. Specifically, we analyse how power-law models can be applied to increase our understanding of signal transduction pathways when there maybe limited prior information. We distinguish between two kinds of power law models: a) *Detailed power-law models*, as a tool for investigating pathways when the structure of protein-protein interactions is completely known, and; b) *Simplified power-law models*, for the analysis of systems with incomplete structural information or insufficient quantitative data for generating detailed models. If sufficient data of high quality are available, the advantage of detailed power-law models is that they are more realistic representations of non-homogenous or crowded cellular environments. The advantages of the simplified power-law model formulation are illustrated using some case studies in cell signalling. In particular, the investigation on the effects of signal inhibition and feedback loops and the validation of structural hypotheses are discussed.

Keywords: Cell signalling, systems biology, power-law models, molecular crowding, inhibition of signal transduction, validation of hypotheses.

Introduction

The mathematical modelling of intracellular interactions is fraught with difficulties, which range from the challenges of measurement, to the assumptions concerning the circumstances and sequence of reactions in a signal transduction pathway. These issues mean that the mathematical modelling of signal transduction pathways is always an approximation in which the art of making appropriate assumptions is of paramount importance. Mathematical models are abstract representations of an underlying biological truth that is so complex that all models are, in a strict sense, wrong. Models are

[1] **Corresponding author:** Olaf Wolkenhauer. Systems Biology and Bioinformatics Group. Department of Computer Science, University of Rostock. 18051 Rostock, Germany.
E-mail: olaf.wolkenhauer@uni-rostock.de Web: www.sbi.uni-rostock.de
Tel.: +49 381 498 75 77 Fax: +49 381 498 75 72

"reduced" and/or "phenomenological" representations of a richer reality, regardless whether this reality is translated into equations by use of mass action principles (Heinrich and Schuster 1996), power laws (Voit 2000) or Markov processes (Van Kampen 2001). The models are "reduced", since from a practical viewpoint, models are usually simplified versions of reality because not all proteins of interest can be considered in experiments. More often than not, there will be interactions and influences that are relevant to the cell function under consideration, but which have to be ignored in the mathematical modelling process. In the same spirit, all pathway models are "phenomenological" because the biophysical nature of molecular interactions is often not understood when modelling biochemical reactions at the detail level of changes in protein concentrations,

The further complication in mathematical modelling is that the technologies and protocols for measurement of biological data records are often (usually) of insufficient duration, accuracy, and spatial and temporal resolution for accurate estimation of model structure and parameters. This, (added to the complexity of intra- and inter-cellular dynamics with the associated assumptions and simplifications), renders all mathematical models approximate, regardless of which of the competing methodologies they are based, be it automata (Heiner et al. 2004), process algebras or formal languages (Errampalli et al. 2004), mass-action principles or power-law representations. Despite the dramatic simplifications of the bio-physical/chemical reality under consideration, mathematical modelling has proven of significant use in illuminating biological mechanisms and guiding experimental investigations. For these reasons it is important that the validity and use of a range of model structures be explored. The aim of this article is to perform such an exploration for a particular form of model - the power-law model - and compare their use with generalised mass action with respect to their "usefulness" in cell signalling systems biology. In this connection, a "good" or "useful" model in systems biology should be:

- An intuitive means to encode hypotheses concerning a system.
- Able to reproduce (fit) experimental observations.
- Predict behaviour not directly observed in experiments.
- Useful in the design of experiments, (e.g. deciding which variables to measure and how).

The vast majority of models for signal transduction pathways are based on nonlinear ordinary differential equations (NODEs). Moreover, these NODEs models are mostly derived or motivated by chemical kinetics (Atkins 2002). The correspondence between the biophysical reality of the cell and its mathematical encoding is reflected in the structure of the rate equations that are used. The terms, variables, coefficients and exponents are justified or explained through the laws of mass action. The 'level' at which a mathematical model is constructed in cell signalling systems biology is therefore related to a description of changes in protein concentration. Alternatively, more detailed models are provided by the analysis of molecular dynamics (Xu 2006 and Kim 2006), and at a yet more granular level, the concepts of quantum chemistry (Haile 1992, Dahl 2005) would be required to include the spatial structure of molecules and details of conformational changes during reactions. Such detailed models and simulations are of such complexity that they are limited to relatively small systems (say a single receptor) and could, at present, not be scaled up to entire pathways. In cell signalling systems biology therefore we therefore choose a higher, less precise, level of modelling where entire pathways and changes of protein concentrations are represented by rate equations. Despite the relative lack of precision, the rate equation approach has proven of great use in the study of regulatory mechanisms and the information transfer that realises cell function (apoptosis, cell differentiation, cell proliferation, cell growth etc.).

The most commonly employed conceptual framework for the construction of rate equations is based on mass-action principles and is commonly characterized by integer-valued kinetic orders (Cornish-Bowden 2004). In this paper however, we focus on the potential advantages of these models when non-integer kinetic orders are involved. In this spirit, the paper is laid out as follows. Section 1 deals with the assumptions under which non-integer kinetic orders occur in signal transduction pathway models. It is shown that the most popular modelling formalisms can be derived from an initial equation by making assumptions about the mathematical and biophysical nature of the systems under consideration. In Section 2 the properties and uses of power-law models are discussed in the context of cell signalling. In particular, the use of power-law models is presented in three practically useful cases:

(i) in simplified models where reaction steps are aggregated, (ii) to represent inhibitory processes, and (iii) as a means of validating hypotheses concerning the pathway structure. The paper closes with some brief conclusions and reflection on further work.

1. Molecular Realism and Modelling Pragmatism

Two contrasting ideas motivate the use of power-law modelling in cell signalling systems biology. The first is a need for a more realistic representation of the cellular environment, and the second is the wish to obtain an intuitive way of representing experimental data. In the first case, positive-valued non-integer kinetic orders emerge naturally as a result of non-homogeneity and molecular crowding within the cell. The second case arises in situations where there is a lack of information about the bio-physical nature of molecular interactions and their environment, and the modeller is required to makes simplifying assumptions. We will examine and give examples of both situations.

1.1. Inhomogeneity of the cellular environment

While it may be assumed that temperature, water balance, pH level, volume and temperature are constant and at a defined level inside the cell, the assumption that molecules float freely around in a well mixed, homogenous gas-phase like environment, is in many cases an unjustified assumption. Various experimental studies support the idea that the inhomogeneity of the environment inside the cell plays an important role in biochemical processes (Kopelman 1986, Luby-Phelps et al. 1987, Elowitz et al. 1999). The existence of a significant number of molecules which do not participate in the biochemical network, but occupy an important fraction of the space inside the cell, alters the dynamics of the system. Under these conditions, the intracellular medium becomes anisotropic and inhomogeneous. The ability to diffuse through such a medium is not the same for all molecules, and will depend on their size and geometry. Molecules, which are small with respect to the size of the obstacles, like metabolites, would be able to percolate between the obstacles easily, while for molecules of the same size or larger than the obstacles, diffusion will be more tortuous (Minton 2001, Schnell and Turner 2004). In this situation, the effective dimension of the space available for reactions and diffusion is fractal and smaller than the original three-dimensional space. The variable diffusion hypothesis has been supported by the work of Kopelman (1986, 1988) and Verkman (2002). In their experiments, they showed how chemical reactions in non-homogenous and crowded media (e.g., the cytosol) are affected by an anomalous diffusion-reaction process that influences in some cases the dynamics of the reactions.

For instance, in a simple homodimeric reaction, which scales with a kinetic order of two in conventional kinetic models, Kopelman (1986) estimated an effective value for the kinetic order of g=3 when the reaction occurs in a channel (one-dimensional system), and g=2.46 when it occurs on a surface (two-dimensional system). The higher the restriction in the dimensionality is, the higher the order of reaction becomes. Therefore, in the case of homodimeric reactions in a crowded intracellular three-dimensional medium, the value for the kinetic order would be expected to vary between two (free diffusion) and 2.46 depending on how the molecular crowding constrains the space available. The higher the crowding is, the bigger the value of the kinetic order in the reaction, with the magnitude of the kinetic order depending upon the species considered and their ability to diffuse in this specific medium.

1.2. The general form of kinetic model

When the assumptions concerning inhomogeneity of the intracellular environment are included, the dynamics of a system of intracellular reactions can be represented using a model composed by a set of nonlinear ordinary differential equations with the following structure:

$$\frac{dX_i}{dt} = \sum_j \sigma_{ij} \cdot \gamma_j \cdot \prod_k X_k^{g_{jk}} \qquad (1)$$

In these equations, γ_j are rate constants, g_{jk} kinetic orders, and σ_{ij} the coefficients of the stoichiometric matrix. Equation (1) can be considered as a general form, since a range different modelling formalisms for biochemical systems emerge from this basic mathematical structure (see Figure 1). For example,

what will be referred to as *conventional kinetic models* (in the context of cell signalling systems biology) are a special case of the above structure in which the kinetic orders are integers. These values will be usually one, but may take the value two if, for example, dimerisation is considered. These values relate directly to the molecularity of the reaction, *m*, i.e., the number of molecules that are assumed to participate in the reaction (as contained in the stoichiometric coefficients of the reaction). Since these values are defined *a priori*, they do not have to be estimated from data, thus allowing an intuitive construction of the rate equations (Cornish-Bowden 1995, Heinrich and Schuster 1996, Fell 1997). The simplicity of this equations set-up and its ease of use is one reason for the success of the kinetic models in the representation of cell signalling. Thus, when in a specific reaction of a pathway a compound appears as reactant, the kinetic order assigned is equal to the stoichiometric coefficient (supposing that the stoichiometric matrix is normalised, containing only the minimum integer numbers possible for each reaction). When a compound does not appear as a reactant in a reaction, the kinetic order is zero such that we can write the conventional kinetic model form:

$$\frac{dX_i}{dt} = \sum_j \sigma_{ij} \cdot \gamma_j \cdot \prod_k X_k^{m_{jk}} \ , \ \forall g_{jk} = m_{jk} \in \{0,1,2\} \tag{2}$$

Where, the kinetic order m_{jk} of a reactant is one when it participates in the interaction, two when dimerisation takes place and zero if it is not involved.

The principal limitation of the conventional kinetic model shown in equation (2) is that the complete structure of the system must be perfectly known, which is usually not the case in signalling pathways. Nonetheless, the approach is commonly used, and special cases are derived from it. For example, a popular special case is obtained by making quasi steady-state assumptions for some components. This leads to *Michelis-Menten type models*. Similar, but more complex equations can be derived using different versions of the quasi steady-state approximation to describe more complex protein-catalysed dynamics (allosteric processes, competitive inhibition, cooperativity and so on). While the quasi-stationary approximation is generally accepted for systems operating in a preferential steady-state (e.g. metabolic pathways), in the context of signal transduction pathways, where the focus is on transient behaviour, these assumptions can be questioned (Cox 1993 and Millat et al. 2006).

1.2. The power-law form of kinetic model

Power-law models follow the structure of Equation 1, by allowing non-integer values for kinetic orders. When the complete structure of interactions is considered, this leads to *detailed power-law models* in which kinetic orders have always real positive values and relate to the effects of molecular crowding and inhomogeneity in intracellular compartments (Savageau 1998). When a simplified version of the system is required (due to incomplete information about the network), then *simplified power-law models* occur, in which positive real-valued kinetic orders are used. Positive values represent the activation, translocation, degradation, while negative real-valued kinetic orders are an intuitive representation for inhibition (Voit 2000). Figure 1, shows how the different modelling formalisms are related.

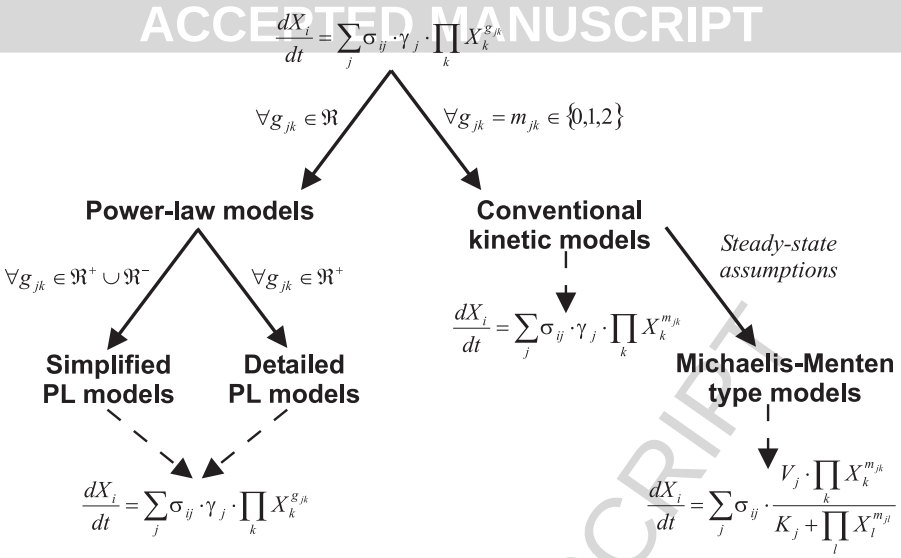


Figure 1. Overview of rate-equation model structures for signal transduction pathways. When the molecularity of reactions is used to assign values to the kinetic orders $(m_{jk} = \{0,1,2\})$, we obtain a conventional kinetic model. Once we obtain this conventional kinetic model, the use of quasi steady-state assumptions lead us to Michaelis-Menten type models. Power-law (PL) models emerge when non-integer kinetic orders are used. If the structure of the network is well-known, detailed PL are derived with kinetic orders always positive real numbers $(g_{ij} \in \Re^+)$. Lack of structural information and the need for simplification and aggregation lead us to simplified PL models with negative real kinetic orders for inhibition $(g_{ij} \in \Re^-)$.

This paper focuses on the use of power-law models in signal transduction pathways and will discuss the use of such models in three examples. For the purposes of these discussions it will be important to distinguish the two avenues that motivate power-law models:

1. Increased realism (accounting for molecular crowding through non-integer kinetic orders).
2. The aggregation of mechanistic detail leading to simplified, easy-to-use, representation of feedback mechanisms (with negative kinetic orders).

With the current quality of the experimental data available for the mathematical modelling of signalling systems, it is difficult to know whether the contribution to the non-integer kinetic orders due to molecular crowding is relevant or not. In a power-law model the number of parameters is higher than in a conventional kinetic model with the same structure and measured states because kinetic orders must be also calculated. This complicates the process of estimating the model parameters. If the data available is limited and noisy, then the larger the set of parameters to be estimated is, the more probable is the chance of finding multiple suboptimal solutions that fit the data equally well (Rodriguez-Fernandez et al. 2006a). These problems of local optimal solutions (Moles et al. 2003), and the more general problem of identifiability (Cobelli and DiStefano 1980), have to be considered carefully and appropriate numerical methods applied.. In the case of local optima, the use of global optimisation methods will increase the possibilities of exploring the parameter space in such a way that suboptimal (i.e. local) solutions are avoided (Rodríguez-Fernández et al. 2006 and Moles et al. 2003). Practical identifiability problems on the other hand can be mitigated by i) increasing the quantity and quality of experimental data available, and ii) the application of optimal experimental design techniques (Balsa-Canto et al. 2006). However, it is also important to note that lack of identifiability can also be a structural feature of the model (Wellstead and Edmunds, 1975) in which case the experimental design must be changed in specific ways.

However, the quality and quantity of data is increasing with innovations in biological measurement and sensing. For example, the emergence of new methods for error reduction (Schilling et al. 2005),

together with new imaging systems like near-infrared (NIR) fluorescence (Picariello et al. 2006), will permit an increase in the quality of quantitative western blots. The generation of massive quantitative data based on ELISA technology (Engvall and Perlman 1971), which is available for an increasing number of proteins, will multiply the number of time points and replicates possible, as well as improving the time resolution and the accuracy in the measurements (Heyman 2006). Methods for the quantification of proteins, based on live cell imaging, are being currently developed and will permit a better estimation of translocation processes and protein-protein interactions (Michalet et al. 2003). Finally, the absolute quantification of proteins and phosphoproteins using mass spectometry and liquid cromatography should be operative in the coming years (Gerber et al. 2003).

With the increasing quality of data and associated computational methods, it is probable that the data with which to estimate the parameters of power law models will improve rapidly. . Nonetheless, as in all model parameter estimation methods, statistical significance tests (Burnham and Anderson 1998) should be used to justify the use of power-law models and to decide whether the quality of the experimental data allows a meaningful distinction between kinetic orders of, say, 1.00 and 1.25. Moreover, it may not be necessary to allow non-integer kinetic orders for every variable if prior knowledge for some of them is available. Given these caveats on the use of power-law models, the next section discusses situations in which they have strong potential and show tangible benefits.

2. Power-law Models of Signal Transduction Pathways

The precise structure of a signalling pathway is often less clear than for metabolic systems. Moreover, the biophysical nature of molecular interactions is complex and often poorly understood even for simple reactions (Atkins 2002). These difficulties oblige the analyst to make simplifying assumptions by, for example, aggregating reactions steps. This need to simplify mechanistic details makes power-law models extremely useful since they are able to accommodate aggregation within them. This simplifying feature of power-law models also extends to the situation where there is sparse evidence about the biophysical nature of interactions. Conventionally in such situations, poor information from experiments is complemented with information from the literature and databases to formulate hypotheses concerning the structure of a mathematical model. In this situation a conventional mass-action type model may appear "realistic"; even though the reactions that it encodes are often partially speculative. However, by aggregating unknown mechanistic details within a power-law model, it is possible to obtain a simple, intuitive mathematical model that is consistent with experimental data.

2.1. Simplification and aggregation

When there is little experimental evidence for the structure of a biochemical network, the aggregation and simplification of models describing the system is a necessity rather than an option. To illustrate this idea, let us consider a pathway integrated by a set of sub-reactions which involve several unknown intermediates states. Consider the case of the simple activation of a certain protein R with a signal S to obtain R^* (Figure 2).

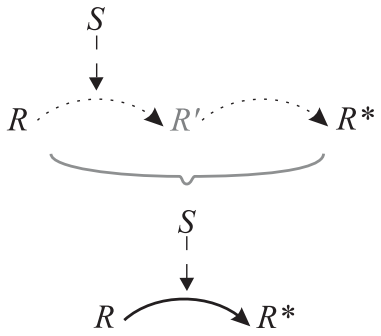


Figure 2. Aggregation of a protein activation process involving an intermediate non-observable state, *R'*.

The value of the signal S can be exactly determined and the concentration of the protein either in its non-activated, R, or in its activated form, R^*, can be measured. For the actual process, there could be an intermediate pre-activated state of protein, R', that cannot be estimated from data and may be unknown. In this case, the strategy is to estimate an empirical rate law for the reaction where, not only the rate constant, but also non-integer kinetic orders are estimated from experimental data:

$$\frac{dR^*}{dt} = \gamma \cdot S^{g_1} \cdot R^{g_2} \tag{3}$$

To illustrate the importance and utility of this form of simplification through aggregation, we turn to the JAK2/STAT5 signalling cascade as described in the paper by Swameye et al. (2003). This cascade is activated through various receptors, including tyrosine kinases, G protein-coupled receptors, and hematopoietic cytokine receptors such as the erythropoietin receptor (EpoR), where EpoR exists as a preformed dimer. Upon binding of the hormone erythropoietin (Epo) the receptor-associated JAK2 is activated through conformational changes of the receptor, transphosphorylates each other and phosphorylates various tyrosine residues in the cytoplasmic domain of EpoR. Subsequently, the latent transcription factor STAT5 is recruited via its SH2 domain to the activated receptor becomes phosphorylated by JAK2, homodimerises and migrates to the nucleus where it initiates the transcription of various target genes. The scheme of the proposed model is depicted in Figure 3.

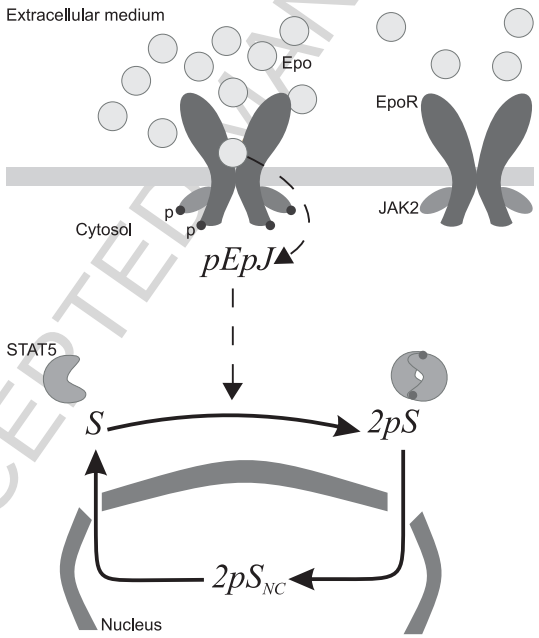


Figure 3. Structure of the JAK2/STAT5 core module. *S* represents non-activated STAT5, *pS is* monomeric phosphorylated STAT5, *2pS* cytosolic dimeric phosphorylated STAT5, and $2pS_{NC}$ the nuclear dimeric phosphorylated STAT5. Activated receptor-kinase complex pEpoR/pJAK2 is denoted *pEpJ* and considered as the input to the system.

A power-law representation that focuses on the core module of the JAK2/STAT5 pathway is discussed here. This includes equations that take account of phosphorylation of STAT5 by the activated Epo receptor, the subsequent dimerisation and translocation to the nucleus, as well as the return to the cytosol after dephosporylation and loss of dimeric conformation. A complete mechanistic model of the processes of STAT5 in the nucleus would include at least three states: the phosphorylated dimerised STAT5, the dimeric phosphorylated STAT5 bound to the regulated genes, and the monomeric dephosphorylated STAT5. Unfortunately, the experimental data available (e.g. Swameye et al. 2003) does not include the information necessary to describe the system at this level of detail, and concentrations of the species inside the nucleus are unknown. This suggests that one could aggregate this chain of processes to a reduced expression. In this spirit, we consider only the dynamics of nuclear

dimeric phosphorylated STAT5, $2pS_{NC}$, since this may be measureable in the near future as experimental techniques develop. With these simplifications, the dynamics of STAT5 in the nucleus will be represented by the following power-law expressions

$$\frac{d2pS_{NC}}{dt} = c_1^{'} \cdot 2pS^{k_1^{'}} - c_2^{'} \cdot \left[2pS(t-\tau)\right]^{k_2^{'}} \tag{4}$$

In the right-hand side of equation (4), the first term represents a single process – namely the translocation of activated dimerised STAT5 into the nucleus. In this case, and provided the effects of anomalous diffusion can be considered imperceptible, the kinetic order $k_1^{'}$ could be made equal to one. In contrast, the second term represents several subsequent processes, and the single kinetic order absorbing all this complexity could have any non-integer value higher than zero (but usually, smaller than two). In this case, we assume the formulation proposed by Swameye et al. (2003) where the signal rate is depending on cytosolic dimeric phosphorylated STAT5, *2pS*, and suggests the existence of a time delay associated with this process. Moreover, we assume that phosphorylation of STAT5 in the receptor, which seems to be a complex process involving several steps, can also be represented with a simple power-law expression using a similar strategy of simplification and aggregation. The dimerisation of pSTAT5 is considered to be much faster than other processes in the signalling pathway. It is therefore neglected as an independent term, and its effect is absorbed by the non-integer kinetic orders representing activation. Thus, the model proposed includes the following states: non-activated STAT5, *S,* cytosolic dimeric phosphorylated STAT5, *2pS,* and nuclear dimeric phosphorylated STAT5, $2pS_{NC}$. The activated receptor-kinase complex pEpoR/pJAK2 is considered as input signal of the system, *pEpJ*. Under the assumptions stated, we arrive at the following mathematical model for the signalling pathway:

$$\frac{dS}{dt} = 2 \cdot \gamma_1 \cdot \left[2pS(t-\tau)\right]^{g_1} - 2 \cdot \gamma_2 \cdot S^{g_2} \cdot pEpJ^{g_3} \tag{5.1}$$

$$\frac{d2pS}{dt} = \gamma_2 \cdot S^{g_2} \cdot pEpJ^{g_3} - \gamma_3 \cdot 2pS^{g_4} \tag{5.2}$$

$$\frac{d2pS_{NC}}{dt} = \gamma_3 \cdot 2pS^{g_4} - \gamma_1 \cdot \left[2pS(t-\tau)\right]^{g_1} \tag{5.3}$$

The proposed model is a simplification of the one proposed in Swameye et al. (2003) because it considers three states for STAT5 instead of four. Translocation of *2pS* to the nucleus is represented as an elementary process without any aggregation. Thus, a kinetic order different to one can only relate to the effect of anomalous diffusion. In the present case, we suppose that this effect cannot be detected with the available data and make the value of g_4 equal to one. We consider that cooperativity could play an important role in the activation of STAT5 by the receptor and allow the kinetic orders g_2 and g_3 to have values higher than one. In the case of the time delay, we assume that the careful estimation done by Swameye et al. (2003) is correct and allow τ to vary only around this value. Finally, preliminary tests suggested that the value of g_1 can be assumed to be one. This reduces the complexity of the model, while at the same time giving a satisfactory fit to the data (data not shown), and therefore allowed the value of this kinetic order to be fixed. Table 1 contains the bounds assigned to the different parameters included in the model.

Table 1. Core module of JAK2/STAT5. Bounds assigned to the parameters.

Parameter	g_1	g_2	g_3	g_4	γ_1	γ_2	γ_3
Lower Bound	1.0	1.0	1.0	1.0	0.01	0.01	0.01
Upper Bound	1.0	2.0	2.0	1.0	10	10	10

In order to estimate the parameters in the model we used the data published in Swameye et al. (2003). These include values in arbitrary units for activated receptor complex (pEpoR), activated STAT5 in the cytosol (pSTAT) and total amount of STAT5 in the cytosol (tSTAT5cyt). For the purposes of comparison between the different data sets, all data were normalised. The data for pEpoR were used as a description of the input signal, and a linear interpolation was generated from the data to describe the intensity of the input signal at any time during the experiment. Additional algebraic equations, reflecting the relation between the measured quantities and the variables, were defined for the model:

$$[\text{pSTAT}] = 2x2pS \qquad [\text{tSTAT5cyt}] = S + 2x2pS \tag{6}$$

The variables on the left side hand represent measured quantities, while the right-hand side represent the variables considered in the model. The final step was to appropriately scale the data. The data available are actually not real quantitative data, because they do not relate to the proportion of protein in the considered state. Additional biological assumptions were used to establish the proportion of protein activated in the peaks of stimulation for both variables *pEpJ* and *2pS*. The initial state of the system, after starvation and before stimulation, can be approximated by assuming that virtually the entire amount of STAT5 was in an inactivated state. It is then possible to assign fixed initial conditions to the states, thus reducing the number of degrees of freedom during model calibration. Under the stated biological assumptions, in the peak of activation after stimulation 85% of the EpoR/JAK2 on the plasma membrane was considered activated and 60% of the STAT5 was supposed to be activated and dimerised.

In order to estimate the model parameters, two experimental data sets obtained in different experimental conditions were used. A multistart local optimisation method indicated the presence of several suboptimal solutions, thus suggesting the use of global optimisation methods (Moles et al. 2003). In this paper, we used the hybrid global optimisation approach recently proposed by Rodriguez-Fernandez et al. (2006b). Figure 4 presents the best fit for one of the experimental data sets used for calibration.

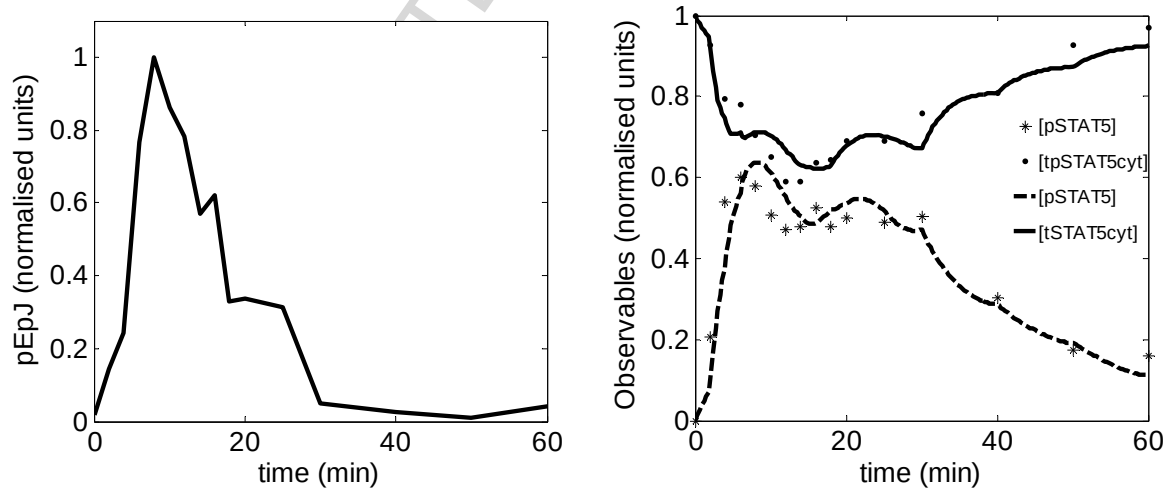


Figure 4. Example of time course data used to estimate the kinetic orders in the JAK2/STAT5 model. Left: measured values of *pEpJ* that were used as an input signal. Right: experimental data (points) and the model fit (lines) obtained with the values estimated for parameters.

The estimated parameters obtained from the optimisation are given in Table 2. The dynamics of the activation, which are represented by γ_2 seem much faster than the other processes described in the model. The kinetic orders g_2 and g_3 have values significantly different to one (respectively, 70% and 30%). Moreover, the difference between g_2 and g_3, which are supposed to be identical and equal to one in a conventional kinetic model, is around 35%. This result supports the belief that the dynamics of the

phosphorylation of STAT5 in the Epo receptor is more complex than the simple kinetic approximation would suggest.

Table 2. Core module of the JAK2/STAT5 pathway. Values of the parameters in the selected solution.

Parameter	g_1	g_2	g_3	g_4	γ_1	γ_2	γ_3	τ
Value	1.00	1.73	1.28	1.00	0.49	4.69	0.15	5.00

The estimated parameters obtained can be used to further refine of the model. Specifically, if an investigation of the mechanisms of reaction is the aim of the model and adequate experimental data are available, then the processes with kinetic orders different to one can be described in more detail. Alternatively some kinetic orders (g_4, g_1) may be fixed as in the conventional kinetic approach to improve the identifiability properties of the model.

2.2. Modelling inhibitory processes

Another interesting and useful property of power-law models is their ability to model inhibition with simplified equations, even when detailed information about the mechanism of inhibition is not available. This can be illustrated with a simple example in which the activation of a protein *R* with a signal *S* is described. Suppose, as illustrated in Figure 5, it is known that a certain protein *I* is able to inhibit this process, through some unknown inhibitory mechanism.

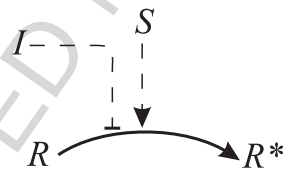


Figure 5. Scheme of a simple signal-driven activation process with inhibition. *R* is activated by the signal protein *S* but the process is inhibited by the regulatory protein *I*.

The power-law model of the biochemical process represented in Figure 6 is:

$$\frac{dR^*}{dt} = V(S,R,I) = \gamma \cdot S^{g_1} \cdot R^{g_2} \cdot I^{-g_3} \tag{7}$$

The inhibitory effect of *I* is denoted by a negative non-integer kinetic order. When negative kinetic orders are used, effects of changes in the value of the considered variable are inversed. In this way, if the concentration of *I* increases, it provokes a decrease in the value of the signalling rate *V(S,R,I)*. In contrast, a decrease in the value of *I* increases the value of the signal rate. Thus:

$$I \uparrow\uparrow \;\Rightarrow\; V(S,R,I) \downarrow\downarrow \quad \wedge \quad I \downarrow\downarrow \;\Rightarrow\; V(S,R,I) \uparrow\uparrow \tag{8}$$

What is described here is the expected effect of an inhibitory protein on the signalling rate, where the intensity of the inhibition is modulated by the value of the negative kinetic order. The higher the value of g_3 is, the stronger the effect of the inhibition becomes. In this way, the value of the kinetic order gives us an idea of the strength of the inhibition by *I*. We have applied this idea to the inhibition of NFκB by RKIP, an interaction that was described and analysed in an interesting paper by Yeung et al. (2001). In Yeung's work, the effects of RKIP on the phosphorylation of IκB proteins by IKK were analysed**.** The authors transfected 293 cells with FLAG-tagged IKK expression vectors with or without an RKIP expression vector. Thirty hours after transfection, cells were either left untreated or were stimulated for 10 min. The results showed that the inclusion of RKIP in the transfection reduced the *in*

vitro IKK activities four- to fivefold in both assays. This observation was interpretted as an inhibition of the cited proteins by RKIP, although the exact molecular mechanism by which this occurs is unknown. Figure 6 shows a simplified model describing the activation of NFκB by IKK and the inhibitory effect of RKIP in the process.

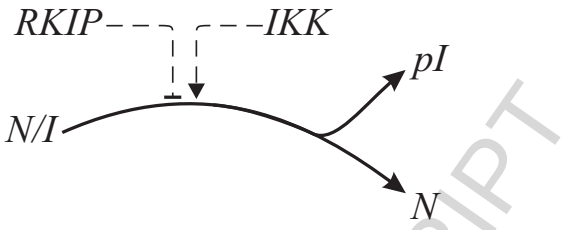


Figure 6. A scheme representing the phosphorylation of IkB (*pI*) by the Ikb kinase (*IKK*). In the process, the complex NFκB/IkB (*N/I*) is broken and NFκB (*N*) is free to translocate to the nucleus where it promotes activation of several genes.

The variables involved represent the IκB kinase, *IKK,* which is the input signal activating the process, the NFκB/IκB complex, *N/I*, phosphorylated IκB, *pI,* NFκB, *N,* and *RKIP*. After activation by *IKK*, IκB is phosphorylated and the complex NFκB/IκB is broken. NFκB can then translocate to the nucleus where it regulates the expression of several genes, such that phosphorylated IκB is degraded. These observations translate into the following equations:

$$\frac{d(N/I)}{dt} = -\gamma \cdot (N/I) \cdot IKK \cdot \left(\frac{RKIP}{0.01}\right)^{-g} \tag{9.1}$$

$$\frac{dpI}{dt} = \gamma \cdot (N/I) \cdot IKK \cdot \left(\frac{RKIP}{0.01}\right)^{-g} \tag{9.2}$$

$$\frac{dN}{dt} = \gamma \cdot (N/I) \cdot IKK \cdot \left(\frac{RKIP}{0.01}\right)^{-g} \tag{9.3}$$

In these equations, the factor 0.01 is used to scale the inhibitory effect of RKIP, which is not significant at concentrations around 0.01 (normalised units). For simplicity, we have considered the contribution of *IKK* and *N/I* to the process as linear, and then assigned to it a kinetic order equal to one. RKIP is considered an inhibitor, and initially we assign a kinetic order minus one to reflect this feature (g = 1). We initially take gamma equal to one (γ = 1). In Figure 7, we compare dynamical simulations of the system with and without RKIP, using as initial condition the switched off configuration of the system *(N/I(*0)= 1*; pI(*0)=*N(*0)=0*)*.

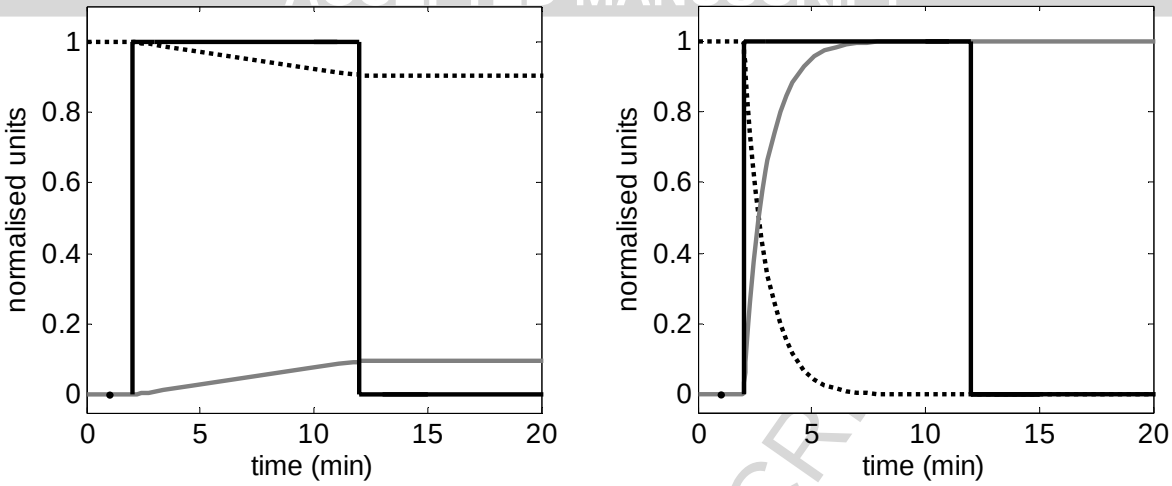


Figure 7. Comparison of a transient stimulation of the system when the inhibition of RKIP is considered. Left: System with a stable concentration of RKIP. Right: System with suppressed RKIP. The solid black lines represent the value of the stimulus *IKK;* the dashed black lines represent *N/I* and solid grey lines represent *pI*.

Figure 8 shows the effect on the model of a pulse of IKK with a normalised intensity of 1.0 between two and twelve minutes (*IKK*(*2:12)*=1.00). The dynamics of the system were simulated for a configuration with RKIP in the left side (*RKIP*=1.00) and without RKIP in the right side (*RKIP*=0.01). It can be seen from the simulations that the activation of the system is very slow in the case of inhibited system (left panel of Figure 8), which provokes a very weak signal for the analysed transient signal. However, in the absence of the inhibitor (right panel of Figure 8), the system reacts rapidly and only five minutes are necessary to obtain a maximum in the activation of the system. Here we can see how the simple power-law expansion used to describe the inhibition of RKIP is able to clearly distinguish the two possible scenarios. Moreover, different values in the kinetic order *g* encode different intensities in the inhibitory strength of RKIP.

In Figure 8 the response of the system with sustained stimulation is studied when the value of the kinetic order *g* is varied from zero (meaning no inhibition by RKIP) to a value of one. In absence of inhibition, the response of the system is fast, and it reaches the maximum activation in only five minutes. When the value of *g* is increase the reaction becomes slower, and from *g*= 0.70 onward, the maximum activation is not reached in the time scale represented (20 times more time than in the case of no inhibition). Therefore, the variation on the parameter *g* allows the strength of the inhibitory effect to be modulated, and can be estimated by fitting real quantitative experimental data.

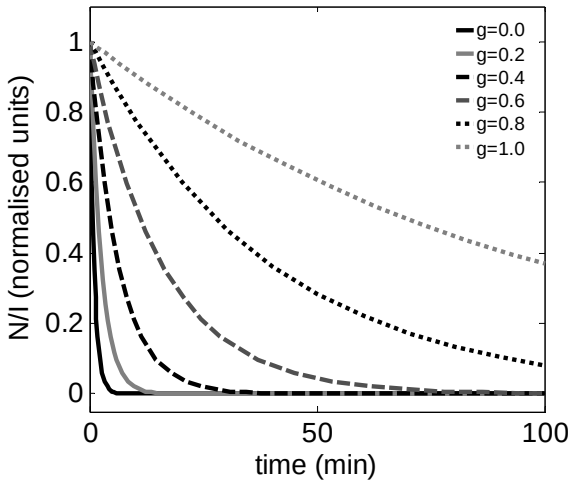


Figure 8. Degradation of *N/I* for a sustained stimulus (*IKK*(0:100)=1.0) and different strengths attributed to the inhibitory effect of RKIP, which are modelled by changing the value of the associated kinetic order (*g*).

2.3. Homogeneity of equations as a tool to validate hypotheses

By modifying the value of the kinetic order in the power-law equation, the properties of a function can vary from the description of an inhibitory process to the description of cooperativity. This allows the analyst an important experimental degree of freedom in the evaluation of different hypotheses concerning the nature of interactions. We illustrate this idea with the following simple example, involving two proteins (X_1 and X_2):

$$V(X_1, X_2) = \gamma \cdot X_1^{g_1} \cdot X_2^{g_2} \tag{10}$$

Suppose the value of X_1 remains approximately constant and equal to one, and the associated kinetic order is also equal to one (g_1= 1.00) - implying linearity of the process with respect to kinetic order. The value of γ is also established as equal to one, and we focus the analysis on the effects of different values for the kinetic order g_2. Depending on the values assigned, the behaviour described by the power-law term changes completely. Negative values for the kinetic order represent inhibition, which means that the value of the function decreases when the value of X_2 is increased. A zero for the kinetic order indicates that the variable does not affect the described process. In the case of positive values of kinetic order, several alternatives are possible. If the kinetic order is equal to one, the system is reproducing a perfectly conventional kinetic behaviour. With values for the kinetic order between zero and one, the equation is representing a saturation-like behaviour in the interval of feasible values for the variable X_2. Finally, with values higher than one, the system models cooperative processes. In Table 3 we summarise the different behaviours reproduced by the function when the value of the kinetic order is modified.

Table 3. Dynamics of the system with respect to values of the kinetic order.

Value g_2	**Behaviour represented**
[-2.0,0.0)	Inhibition
0.0	No interaction
(0.0,1.0)	Saturation (approx.)
1.0	Perfect kinetic
(1.0,2.0]	Cooperativity

Figure 9 shows how the properties of the function change drastically for different values of the kinetic order included in the different intervals of Table 3.

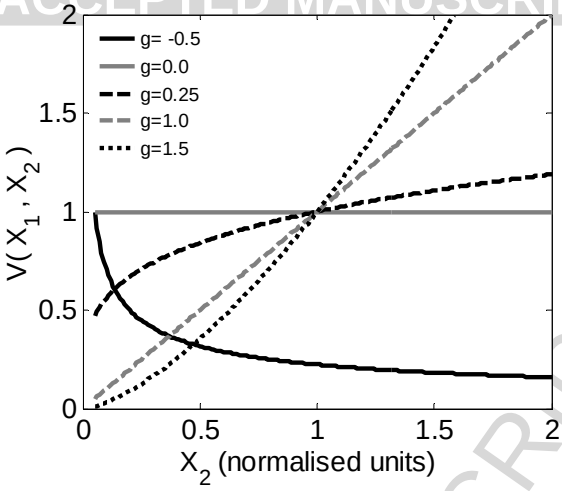


Figure 9. Dependence of the function $V(X_1, X_2) = \gamma \cdot X_1^h \cdot X_2^g$ on changes in the value of X_2. Different representative values for g are considered. All equations represented have the same structure and the same values for parameters other than g (X_1=1.00, g_1=1.00 and γ =1.00). The exception is the case of inhibition, where the variable X_2 was adequately normalised to have values for the function in the same range than the other cases ($X_2' = X_2/0.05$).

All equations simulated in Figure 9 have exactly the same structure as previously described, but different behaviours appear clearly in the figure. Thus inhibition (g= -0.5) provokes a significant decrease in the values of the function while the value of X_2 increases. In case of positive kinetic orders, the function describes a saturation-like curve when a value lower than one is considered for g, but increases faster than the perfect kinetic case when higher values are considered.

To illustrate how the regularity and flexibility of power-law models helps in pathway characterisation, consider a very simple network of four interacting proteins *X, Y, W* and *Z* in which the exact structure of the interactions is unknown (Figure 10). We focus our attention on the dynamics of the protein *X* that is activated in the process, while the contributions of the other variables in the process are unknown.

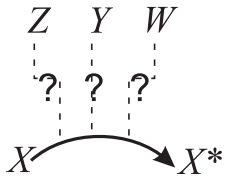


Figure 10. Graphical representation of a network for which the structure is unknown. *X* is activated in the process, but the precise role of *Z*, *Y* and *W* has not been established.

The differential equation model describing the process is:

$$\frac{dX}{dt} = \alpha \cdot X^{g_1} \cdot Y^{g_2} \cdot W^{g_3} \cdot Z^{g_4} \qquad (11)$$

This formulation is not based on any previous information about the system, and any possible variable that might take part in the network is included in the equation. Likewise, there is no information about the possible values of the kinetic orders, which could be even zero, indicating no participation in the process. Using data fitting techniques, experimental data could be used to estimate preliminary values

or feasible intervals for the parameter values (see Veflingstad et al. 2004). Let us suppose we obtain the following solution:

$$\frac{dX}{dt} = -0.145 \cdot X^{1.005} \cdot Y^{1.450} \cdot W^{0.001} \cdot Z^{-0.250} \tag{12}$$

The solution per se provides useful structural information about the nature of an interaction that was previously unknown. In particular, the kinetic order associated with *X* is near 1, and thus represents the usual participation of a protein in its own activation. The cases of *Y, W* and *Z* are more interesting. The kinetic order of *Y* is positive indicating that *Y* activates the process, while the kinetic order associated to *Z* is negative which indicates that this protein inhibits the described process. In the case of *W*, the kinetic order is almost identical to zero, indicating that contrary to the original hypothesis, this protein does not participate in the interaction. These conclusions from Equation 12 are depicted in Figure 11.

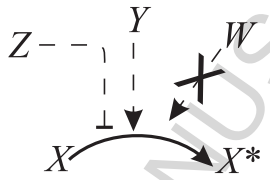


Figure 11. Graphical representation of a network after an analysis using power-law modelling and experimental data. *Y* act as a signal activating *X,* while *Z* inhibits the process. After parameter estimation, the kinetic order associated to W was estimated to be near zero, which means that the effect of *W* on the activation of *X* can be discarded.

This "blind" investigation on the structure of an interaction, based only on the available data, would not be possible without the absolute homogeneity of the power-law equations. We now illustrate this feature of power-law modelling for the inhibition of Ras/Raf1/MEK/ERK pathway by RKIP. The aim of the experiment is to determine whether the previously described inhibitory behaviour of RKIP in Ras/Raf1/MEK/ERK (Yeung et al. 1999) appears also in MCF10A cells, a spontaneously immortalised, but nontransformed human mammary epithelial cell line (Soule et al. 1990). A simple model describing the activation of this pathway and the potential role of RKIP is shown in Figure 12.

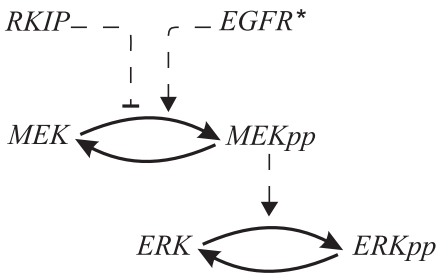


Figure 12. Model describing the possible inhibition of RKIP in Ras/Raf1/MEK/ERK pathway. In this simple model we consider only the phosphorylation of MEK and ERK. Previous steps of the pathway are ignored. The effect of the signal used in the experiments is modelled with the variable *S*.

The dynamics of the system are described by the following equations:

$$\frac{dpMEK}{dt} = \gamma_1 MEK \cdot EGFR^* \cdot RKIP^{-g_1} - \gamma_2 pMEK \cdot ERK \quad (13.1)$$

$$\frac{dpERK}{dt} = \gamma_2 pMEK \cdot ERK - \gamma_3 pERK \quad (13.2)$$

$$MEK = K_1 - pMEK \quad (13.3)$$

$$ERK = K_2 - pERK \quad (13.4)$$

The inhibitory effect of RKIP is described by the kinetic order g_1, which must be estimated from experimental data. We estimate the values for the parameters using quantitative data produced with ELISA kits that describe the dynamics of Ras/Raf1/MEK/ERK pathway in the considered cell line after stimulation for normal concentration of RKIP (*RKIP*=1.00), overexpression RKIP (*RKIP*=3.00), and with repression (*RKIP*=0.10). We focussed our attention on the value of g_1, which describes the structural property that we are considering. The interval of values allowed for g_1 was between zero (no interaction), and two (strong inhibition). The value calculated was g_1= 0.00. That is, we can assume that the inhibition by RKIP can be discarded in models describing this pathway for the considered cell line:

$$\frac{dpMEK}{dt} = \gamma_1 MEK \cdot EGFR^* - \gamma_2 pMEK \cdot ERK \quad (14.1)$$

Once this structural hypothesis is discarded for this cell line, the model can be refined to improve the description in accordance with available data.

3. Conclusions

The rationale for using non-integer kinetic orders in models of biochemical networks comes from either consideration of molecular crowding in inhomogeneous environments ("detailed power-law models"), or where the absence of detailed mechanistic information or measured data necessitates an aggregation of details ("simplified power-law models"). Detailed power-law models are a future perspective given the limited technologies currently available to generate quantitative, sufficiently rich and accurate time series datasets. The situation for simplified power-law models is however different. The detailed structure of the interactions in the majority of the signal transduction pathways is an open question, and the experimental data available are usually not sufficient to estimate parameters in large models with an acceptable level of confidence. In this context, the need for simplification is a reality, and simplified power-law models can make an interesting contribution to the modelling workflow in which mathematical models are iteratively improved.

The main problem associated with the use of power-law models is particular parameter estimation of nonlinear models. Assuming that structural identifiability is guaranteed, two types of problems may appear: several suboptimal solutions and different but equivalent solutions. While identifiability is a general problem for all kind of models, it is more evident when power-law models are considered. The question is then whether the apparent increase in realism justifies the additional number of parameters that we introduce in power-law models. There is no simple answer to this question, but some clear ideas exist. First, a power-law model is not necessary when a kinetic model with similar level of description is able to describe the dynamics of a system with sufficient accuracy. This is evident in the case of very detailed mechanistic models where the complete network of interactions is considered. In detailed models the number of parameters to be estimated is much higher in the power-law version than in a conventional kinetic model. However, power-law models become a realistic alternative when simplified models are proposed.

Second, the use of a power-law model does not imply that all kinetic orders in a model need to take non-integer numbers. Using prior knowledge and constraints on the parameter values can significantly reduce the identifiability problem. Parameter estimation in biological pathway modelling is in any case a "supervised" process and for the remaining kinetic-orders, an iterative process can be implemented. In addition, although not considered here, statistical tests on the confidence intervals of kinetic orders

and other approaches based on information theory could be used to decide on the use of non-integer kinetic orders (Kuhn 2005, Liebermeister et al. 2005).

To conclude our view is that power-law models are a useful intuitive strategy for the translation of experimental evidence into a simplified model. The ability of these models to reproduce complex interactions enables the modeller to study general systemic properties of the system (including amplification, inhibition strength, feedback loops) without prior knowledge. In this sense, the initial use of a power-law model provides a strong basis for the development of more refined mechanistic models.

Acknowledgements

This work was supported by the European Commission 6th Framework program and as part of the COSBICS project under contract LSHG-CT-2004-512060 (www.sbi.uni-rostock.de)

The authors appreciate comments by Dr. Christian Fleck and Prof. Dr. Jens Timmer from the University of Freiburg, Germany for discussions. PD Dr. Ursula Klingmüller, Dr. Andrea C. Pfeifer and Julie Bachmann from the German Cancer Research Centre (DKFZ Heidelberg, Germany) provided information about the JAK2/STAT5 pathway. Prof. Walter Kolch and Dr. Oliver Rath from the Beatson Institute of Cancer Research (Glasgow, UK) provided data and information about the role of RKIP in the NFkB and MAPK pathways.

References

Atkins, P., de Paula, J. (2004) Atkins' Physical Chemistry (7th edition). *Oxford University Press*.

Cobelli, C., and DiStefano, J.J. 3rd (1980) Parameter and structural identifiability concepts and ambiguities: a critical review and analysis. *Am. J. Physiol*. 239(1):7-24.

Cornish-Bowden, A. (2004) Fundamentals of Enzyme Kinetics (3rd edition). *Portland Press, London*.

Cox, B.G. (1993) Modern Liquid Phase Kinetics. *Oxford University Press*.

Balsa-Canto, E., Rodríguez-Fernández, M., Alonso, A.A., Banga, J.R. (2006) Computational design of optimal dynamic experiments in systems biology: a case study in cell signaling. *1st Understanding and Exploiting Systems Biology in Bioprocesses and Biomedicine (in press)*.

Burnham, K.P., and Anderson, D.R. (1998) Model selection and inference: a practical information-theoretic approach. *Springer-Verlag, New York.*

Dahl, S.G., Sylte, I. (2005) Molecular modelling of drug targets: the past, the present and the future. *Basic Clin. Pharmacol. Toxicol*. 96(3):151-5.

Elowitz, M.B., Surette, M.G., Wolf, P.E., Stock, J.B., Leibler, S. (1999) Protein mobility in the cytoplasm of Escherichia coli. *J. Bacteriol.* 181(1):197-203.

Engvall, E. and, Perlman, P. (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* 8(9):871-4.

Errampalli, D.D., Priami, C., and Quaglia, P. (2004) A Formal Language for Computational Systems Biology**.** *OMICS: A Journal of Integrative Biology* Vol. 8(4):370 -380.

Fell, D. (1997) Understanding the Control of Metabolism. *Portland Press, London*.

Gerber S.A., Rush J., Stemman O., Kirschner M.W., Gygi S.P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci.* 100(12):6940-5.

Haile, J. (1992) Molecular Dynamics Simulation: Elementary Methods. *John Wiley & Sons, Inc. New York.*

Heiner, M., Koch, I. and Will, J. (2004) Model validation of biological pathways using Petri nets - demonstrated for apoptosis. *Biosystems* 75, 15-28.

Heyman J. (2006) Quantification of Activated Signal Transduction Proteins Using Fast Activated Cell-based ELISAs (FACE™). *Nature Application Notes*. doi:10.1038/an1562.

Heinrich, R., Schuster, S. (1996) The Regulation of Cellular Systems. *Chapman and Hall, New York*.

Kim, S.K., Jacobson, K.A. (2006) Computational prediction of homodimerization of the A(3) adenosine receptor. *J. Mol. Graph. Model*. [Epub ahead of print]

Kopelman, R. (1986) Rate processes on fractals: theory, simulations and experiments. *J. Stat. Phys*. 42:185-200.

Kopelman, R. (1988) Fractal Reaction Kinetics. *Science* 241:1620-1626.

Kuhn, E., Lavielle, M. (2005) Maximum likelihood estimation in nonlinear mixed effects models. *Computational Statistics & Data Analysis* 49:1020-1038.

Liebermeister, W., Baur, U., Klipp, E. (2005) Biochemical network models simplified by balanced truncation. *FEBS Journal* 272 4034-4043.

Luby-Phelps, K., Castle, P.E., Taylor, D.L., Lanni, F. (1987) Hindered diffusion of inert tracer particles in the cytoplasm of mouse 3T3 cells. *Proc. Natl. Acad. Sci.* 84(14):4910-3.

Michalet X., Kapanidis A.N., Laurence T., Pinaud F., Doose S., Pflughoefft M., Weiss S. (2003) The power and prospects of fluorescence microscopies and spectroscopies. *Annu. Rev. Biophys. Biomol. Struct.* 32:161-82.

Millat, T., Bullinger, E., Rohwer, J., Wolkenhauer, O. (2006) Approximations and their consequences for dynamic modelling of signal transduction pathways. *Math. Biosciences (in press)*.

Minton, A.P. (2001) The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiololgical media. *J. Biol. Chem.* 276: 10577-10580.

Moles, C.G., Mendes, P., and Banga, J.R. (2003) Parameter estimation in biochemical pathways: a comparison of global optimization methods. *Genome Research* 13(11):2467-2474.

Newhouse, J.S., Kopelman, R. (1988) Steady-state chemical kinetics on surface clusters and islands: Segregation of reactants. *J. Phys. Chem*. 92:1538-1541.

Picariello L., Carbonell Sala S., Martineti V., Gozzini A., Aragona P., Tognarini I., Paglierani M., Nesi G., Brandi M.L., Tonelli F. (2006) A comparison of methods for the analysis of low abundance proteins in desmoid tumor cells. *Analytical Biochemistry* 354(2): 205-212.

Rodriguez-Fernandez, M., Egea. J.A. and Banga, J.R. (2006a) Novel Metaheuristic for Parameter Estimation in Nonlinear Dynamic Biological Systems. *BMC Bioinformatics* 7:483.

Rodriguez-Fernandez, M., Mendes, P., Banga, J.R. (2006b) A hybrid approach for efficient and robust parameter estimation in biochemical pathways. *Biosystems*, 83 (2-3), 248-265.

Savageau, M.A. (1998) Development of fractal kinetic theory for enzyme-catalysed reactions and implications for the design of biochemical pathways. *Biosystems* 47(1-2):9-36.

Schilling M., Maiwald T., Bohl S., Kollmann M., Kreutz C., Timmer J., Klingmüller U. (2005) Computational processing and error reduction strategies for standardized quantitative data in biological networks. *FEBS J.* 272:6400-6411.

Schnell, S., Turner, T.E. (2004) Reaction kinetics in intracellular environments with macromolecular crowding: simulations and rate laws. *Prog. Biophys. Mol. Biol.* 85(2-3):235-60.

Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson Jr., W.D., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F., Brooks, S.C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.* 50: 6075–6086.

Swameye, I., Mueller, T.G., Timmer, J., Sandra, O., Klingmüller, U. (2003) Identification of nucleocytoplasmic cycling as a remote sensor in cellular signalling by data-based modelling. *Proc. Natl. Acad. Sci.* 100:1028-1033.

Van Kampen, N.G. (2001) Stochastic Processes in Physics and Chemistry (2nd edition). *Elsevier, Amsterdam*.

Veflingstad, S.R., Almeida, J., Voit, E.O. (2004) Priming nonlinear searches for pathway identification. *Theoretical Biology and Medical Modelling* 1:8 doi:10.1186/1742-4682-1-8.

Verkman, A.S. (2002) Solute and macromolecule diffusion in cellular aqueous compartments. *Trends Biochem. Sci*. 27(1):27-33.

Voit, E.O. (2000) Computational Analysis of Biochemical Systems. A Practical Guide for Biochemists and Molecular Biologists. *Cambridge University Press,UK*.

Wellstead, P.E. and Edmunds, J.M. (1975) Least Squares Identification of Closed-Loop Systems, *Int. J. Control*, 21: 4-15.

Xu, Y., Luo, X., Shen, J., Zhu, W., Chen, K., Jiang, H. (2006) Molecular dynamics of nicotinic acetylcholine receptor correlating biological functions. *Curr. Protein Pept. Sci.*7(3):195-200.

Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K.D., Rose, D.W, Mischak, H., Sedivy, J.M., Kolch, W. (2001) Raf kinase inhibitor protein interacts with NF-kappaB-inducing kinase and TAK1 and inhibits NF-kappaB activation. *Mol. Cell Biol*. 21(21):7207-17.

Yeung K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K.D., Rose, D.W., Mischak, H., Sedivy, J.M., Kolch, W. (1999) Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature* 401(6749):173-7.